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The results show that

1. the replicative capacity of HIV-2, like HIV-1, correlates with the severity of immunodeficiency;

2. dual tropism for monocyte/macrophages and CD4+ T lymphocytes is a general property of HIV isolates. HIV can establish a persistent, nonproductive infection in monocyte/macrophages indicating that these cells may serve as infectious resrvoir in the infected individual. Passage of HIV through macrophages may contribute to the biological diversity of virus isolates;

3. molecular clones of a rapid/high HIV-1 isolate may have restricted replicative capacity compared to the genetically heterogenous isolate. Transfection of the molecular clones into different cell types may yield progeny viruses with different biological phenotypes. In cotransfection experiments a mixture of three clones with restricted replicative capacity yielded progeny with rapid/high phenotype. In this population recombination as well as complementation could be demonstrated. The results show that complementation between different coexisting variants may influence the biological phenotype of a viral population;

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cont. 19. ABSTRACT

4. the pattern of neutralization of autologous virus is similar in HIV-1 infected humans and SIV_{sm} (sooty mangabey origin) infected macaque monkeys. In both cases, variant viruses resistent to neutralization by autologous sera emerge during the entire course of infection. The ability to produce neutralizing antibodies to emerging autologous variant viruses appears to correlate with the degree of immunodeficiency in the host. Antigenic changes are accompanied by changes in the biological properties of HIV-1 and SIV_{sm} during the pathogenic process in the human and macaque host, respectively. In contrast, HIV-2 infected humans and experimentally infected macaques are both able to neutralize autologous virus. In macaques, where HIV-2 infection is nonpathogenic, no variation in biological properties of sequential HIV-2 isolates can be observed suggesting that variability and pathogenicity of the virus are closely related properties;

5. virus neutralizing epitopes in the HIV-1 and HIV-2 envelope roughly coincide as demonstrated by use of synthetic peptides. The HIV-2 V3 region was found to carry broadly cross-reactive neutralizing epitopes that could be mapped to the central and C-terminal portion of the V3 region. Using two deletion sets of peptides and human polyclonal and neutralizing mouse monoclonal antibodies, two distinct antigenic sites were identified, one corresponding to the conserved motif Phe-Hys-Ser (aa 315-317), and another in proximity of the C-terminal cysteine (Trp-Cys-Arg, aa 329-331). One monoclonal antibody showed dependence on both sites, signifying that the sites can interact to represent a local discontinuous antigenic site;

6. broad neutralizing activity in the mother's serum against primary HIV-1 isolates (i.e. cross-reactive antibodies) may protect the child from HIV-1 infection. Comparison of V3 sequences from ten HIV-1 infected mother-child pairs showed that the infants, in contrast to the mothers, harbour homogenous virus populations. The transmitted virus did not show any characteristic molecular features and represented either a major or minor population of the mother.

New methodologies introduced:

1. a simple, sensitive and specific polymerase chain reaction (PCR) protocol for the detection of HIV-1 in PBMC of infected individulas;

2. direct sequencing of PCR products immobilized on magnetic beads in an automated laser fluorescent (A.L.F.) sequencing apparatus.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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Erling Norrby Oct. 26, 1992

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INTRODUCTION

The studies described herein have focused on HIV variability, in particular on biologic and antigenic variability and its relevance for pathogenesis and for development of means for immune intervention. During the third year of the grant period we have intensified studies on molecular variability of HIV-1 and attempted to molecularly map biologic and antigenic determinants. Biologic characterization involved tests for replicative capacity, cytopathic effect and monocyte tropism of virus isolates and molecular clones, whereas antigenic characterization involved virus neutralization in autologous as well as heterologous system. By use of synthetic peptides, epitopes for virus neutralization and antibody dependent cellular cytotoxicity (ADCC) have been mapped in the envelope of HIV-1 and HIV-2. Variable region 3 (V3) of the HIV-1 envelope has been studied in relation to biological and antigenic variation of HIV-1 isolates and in relation to mother-to-child transmission of HIV-1.

Previous studies in our laboratory (1, 2) have shown that naturally occurring HIV-1 variants have distinct biologic features that correspond to the severity of HIV-1 infection. Virus from asymptomatic carriers or individuals with mild disease has been shown to replicate slowly and inefficiently in the patients' peripheral blood mononuclear cell (PBMC) cultures. Attempts to passage these viruses in CD4 positive cell lines usually failed or resulted in transient replication only. In contrast, viruses from patients with severe immunodeficiency replicated rapidly and efficiently in PBMC as well as in cell lines. Hence the designation slow/low and rapid/high, respectively. These two groups of viruses can also be distinguished by the type of cytopathogenicity exerted in PBMC (2). Rapid/high viruses are characterized by extensive syncytia formation, whereas syncytia are rarely seen with slow/low viruses. Instead, cultures infected with slow/low viruses show signs of cell death or no cytopathic changes at all. These results have been confirmed and extended by several laboratories world wide (for review see 3, 4, 5) and indicate that the biologic characteristics of the virus seem to play a key role in pathogenesis.

Primary HIV infection is accompanied by viremia which is rapidly cleared concomittant to seroconversion and development of isolate specific neutralizing antibodies (6). It seems therefore that neutralizing antibodies - probably in conjunction with cellular immunity - play a crucial role in clearing the initial viremia. The question arises why then virus replication continues in the course of HIV infection? Is the virus replicating at a later stage different from the virus replicating early during HIV infection? If so, can the immune system cope with the new variants? To approach these questions, in our studies

we have focused on the role of neutralizing antibodies and antibodies mediating cellular cytotoxicity reactions (ADCC). These studies provide the basis for our attempts to identify critical target immunogens for vaccine development.

In collaboration with Dr G. Biberfeld's group we have evaluated different forms of immune interventions in HIV-2 (7) and SIV_{sm} infected cynomolgus macaque monkeys (8). It was demonstrated that infection with the non-pathogenic HIV-2 protects against the pathogenic consequences of a subsequent SIV_{sm} infection (9) and that immunization with inactivated HIV-2 prevents infection with homologous virus in these animals (10). Use of the macaque model for vaccine studies, prompted us to explore autologous neutralizing antibody production, both in the pathogenic SIV_{sm} and the non-pathogenic HIV-2 infection. The kinetics of neutralizing antibody production as well as the extent of cross-reactivity over time are key questions also in experiments involving passive transfer of antibodies. These experiments demonstrated that transfer of serum from asymptomatic SIV_{sm} infected animals or from animals hyperimmunized with HIV-2 can prevent infection with homologous virus (11). In the same system we are currently evaluating the possiblities for establishment of a protective immunity by immunization with synthetic peptides representing selected regions of the glycoproteins of HIV-2 and SIV_{sm}. In the selection of peptides for use in these studies advantage was taken of the knowledge of important immunoprotective domains in the HIV-1 glycoproteins including the loop structure in the variable region 3 (V3) region of the viral envelope (V3-loop) (cf. ref. 12).

Furthermore, to elucidate the molecular mechanisms involved in mother-to-child transmission of HIV-1 we compared V3 sequences from ten HIV-1 infected infants to sequences from the corresponding mothers. In contrast to the highly heterogenous virus populations of the mothers, children harboured homogenous virus populations. Based on similar findings in three mother-child pairs, Wolinsky et al. (13) have recently suggested that one single genotype is selectively transmitted from mother to child. The marker indicating selection was an N-linked glycosylation site, immediately amino terminal to the V3 loop. This marker, while present in the mothers but missing from the children in Wolinsky's material, it was present in nine of ten children tested in our experiments. These studies are ungoing, and much of the continuation is described in our new grant proposal.

METHODOLOGY

Biological characterization of virus isolates: 1) replication in T-lymphoid and monocytoid cell lines. First, peripheral blood mononuclear cells (PBMC) are infected with 5-20.000

cpm reverse transcriptase (RT) activity and 7-10 days later, when cultures are RT positive, $1x10^6$ PBMC are cocultivated with $3x10^6$ cells of each of the cell lines, Jurkat, Jurkat-tat, U937 clone 2 , CEM and/or HUT-78 (2). 2) Cytopathogenicity is scored in PBMC cultures 7-10 days postinfection (2). 3) Replication in fresh monocyte/macrophage cultures. Monocyte cultures are prepared by seeding $2.5x10^7$ PBMC into $25cm^2$ plastic culture flasks in RPMI medium supplemented with 10% heat-inactivated pooled HIV-1 negative human serum and 20% fetal calf serum (FCS). After 5 days at 37°C the cultures are extensively washed with PBS to remove nonadherent cells and maintained thereafter in RPMI medium with 20% FCS (14). Virus replication is followed by measuring HIV-Ag (p24) (15) and RT activity (16) in culture supernatants.

Virus neutralization assay: Virus titrations are done in PBMC according to the method described by McDougal et al. (17) and modified by Albert et al. (6). In short, virus aliquots are diluted in medium, six fivefold dilution steps starting with a 1:5 dilution. First, 75µl of each virus dilution was added to five parallel wells of a round-bottom 96-well culture plate (Nunc, Roskilde, Denmark) and, second, $1x10^5$ PHA-P stimulated blood donor PBMC in 150 µl medium was added to each well. The culture medium is changed/plates washed on each of the following 3 days and on day 7 a 100 µl sample from each well is analyzed in an in-house HIV-Ag ELISA assay (ref. 15 for HIV-1 and ref. 18 for HIV-2). The ID-50 is defined as the reciprocal of the virus dilution resulting in 50% positive wells (Reed-Muench calculation).

Neutralization assays are run simultaneously with each virus titration using three virus dilutions, 1:5, 1:25 and 1:125, for each serum dilution. Five twofold dilution steps of sera, starting with a 1:10 dilution, are added in triplicate (75µl each) to 96-well culture plates. Virus (or medium) is then added in an equal volume and the plate incubated for 1 h at 37°C. Subsequently, 10^5 PHA-P stimulated PBMC in 75µl are added and the plate further incubated overnight. Subsequent washing and testing as above. The negative virus controls consist of two wells with virus but no cells to check the washing procedure and five wells with cells but no virus. Sera from two asymptomatic homosexual men with high-titer neutralizing antibodies against $HIV\text{-}1_{IIIB}$ are used as positive serum controls. All sera and virus isolates obtained from one patient are assayed simultaneouly. The neutralizing titer of a serum is estimated at virus dilution(s) containing 10-50 ID-50 and defined as the reciprocal of the highest serum dilution giving a complete (or 80%) reduction in absorbance value in the HIV-Ag assay as compared to the mean +2SD of five negative wells containing cells only.

We have recently looked into the effectiveness of our washing procedures to remove anti-gag antibodies and whether anti-gag antibodies, if present, would interfere with the ELISA readout of the neutralization assay. Details of these experiments will be described in a manuscript (Albert et al. preliminary manuscript, enclosed). In short, neutralization assays carried out by testing different washing procedures in parallel, show that the presence of anti-gag antibodies in the neutralization assay proper have no influence on the neutralizing titer of the sera. Anti-gag antibodies may be detectable or undetectable at the time of readout, the neutralizing titers are the same.

ADCC: the test was performed as described previously (19). In short, U937 clone 2 cells chronically infected with HIV-2SBL$_{6669}$ were used as target cells. PBMC were derived from normal healthy donors and collected by density centrifugation on Ficoll-Isopaque, and adherent cells removed by the scrubbed nylon wool technique (20). ^{51}Cr-labeled target cells ($1x10^4$) and isolated lymphocytes as effector cells ($2x10^5$) were mixed with serum dilutions. Supernatant was harvested after 3 hours and released radioactivity was calculated.

Peptide synthesis. The peptides were synthesized in 30-70 mg quantities by the method of simultaneous multiple-peptide synthesis (21) and were cleaved with liquid hydrofluoride in a 24 vessel apparatus (22). Their composition was controlled by amino acid analysis and their purity by high performance liquid chromatography characterization using a reverse phase Vydac C4 column.

Peptide-ELISA. Peptides dissolved in 0.1 M carbonate-bicarbonate buffer pH 9.3 were added to microtiter plates in a final amount of 1µg/well. After incubation at room temperature over night the plates were washed with PBS and then blocked for one hour at room temperature with 0.5% bovine serum albumin in PBS. After removal of blocking buffer the test was run as previously described (23).

Western blotting was performed as previously described (24).

DNA preparation. DNA for PCR amplification was prepared directly from uncultured patient's PBMC (25) and from virus isolates after one passage on PBMC from healthy blood donors (26). $2-4x10^6$ PBMC were resuspended in PCR lysis buffer (10mM Tris-HCl pH 8.3, 1mM EDTA, 0.5% NP40, 0.5% Tween 20 and 300µg/ml proteinase-K) at a concentration of 10^6 cells/100µl buffer and digested with proteinase-K overnight at 37°C. The proteinase-K was then inactivated for 15 min at 94°C.

RNA extraction and c-DNA synthesis. HIV-1 RNA was extracted from the serum as described (27). In short, oligo (dT)25-coated magnetic beads (25µl; Dynal AS, Norway) were used to extract viral genomic RNA from 25µl serum. The extracted RNA (20µl) was immediately reverse transcribed into cDNA using the primer JA12 (25), which is complementary to the V3 region of the gp120 env gene of HIV-1. The reverse transcription mixture (30µl) contained 50mM Tris-HCl pH 8.3, 8mM MgCl2, 30mM KCl, 10mM DTT, 1.7mM of each deoxynucleotide (Pharmacia, Uppsala, Sweden), 0.5 µM primer JA12, 3 units MoMuLV reverse transcriptase (Pharmacia) and 16 units RNA guard (Pharmacia). Reverse transcription was performed for 1 hour at 37°C. Sera from blood donors and from HIV-1 infected patients were included as negative and positive controls, respectively.

Polymerase chain reaction. DNA (10µl) or cDNA (5µl) samples were amplified by PCR with nested primers specific for the V3 (25, 26) or other (28) regions of the gp120 env gene. Briefly, the samples were first amplified for 24 cycles with the outer primers, then 1/10 (5µl) of the product from the first PCR reaction was amplified for 30 cycles with the inner primers. Negative controls were included in each run and consisted of lysis buffer alone and samples from healthy blood donors. Positive controls consisted of HIV-1 infected cells diluted in uninfected cells to contain 10 HIV-1 viral DNA copies.

Cloning. The PCR product amplified by the inner primers was purified using the QIAGEN spin 20 column (DIAGEN, Düsseldorf, Germany) and dissolved in 20µl Tris-EDTA. The PCR fragments were treated with Klenow fragment of DNA polymerase I (Pharmacia) as well as T4 polynucleotide kinase (Pharmacia). After precipitation with ammonium acetate approximately 1/10 of purified PCR product was ligated with 50ng *Sma*I digested pUC18 vector (Pharmacia) using the DNA ligation system (Amersham, Buckinghamshire, UK). The ligated vector was used to transform the competent cell JM105 (Pharmacia) by treatment at 42°C for 90 sec. Positive colonies were picked, resuspended in 10µl PCR-buffer (10mM Tris-HCL pH 8.3, 50mM KCl and 0.01% Tween) and treated at 95°C for 5 min. To confirm the presence of the insert in the plasmid, amplification with pUC18 specific primers was performed.

DNA sequencing. The PCR product amplified by the inner primer was used for direct solid-phase DNA sequencing as described (29, 26). Briefly, a further amplification step of the first PCR product (diluted 1/100) was performed with the primers RIT124 and RIT128. The amplified product was then purified by immobilization on magnetic beads (Dynabeads M280-Streptavidin, Dynal AS) and subsequently denatured to obtain single-stranded DNA. Fluorescent primers (RIT129 or RIT43; Universal Froward Primer

[Pharmacia] for the clones) were used for the sequencing reaction of both DNA strands; the products was then loaded on a 6% polyacrylamide gel in an automated laser fluorescent (A.L.F.) sequencing apparatus (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). Each sample was sequenced twice. Computer analysis was performed with the program "Treealign" (30).

RESULTS

Biological properties of HIV-2 isolates.

Whether the biological properties of HIV-2 isolates are similar to those of HIV-1 is a particularly important question, since HIV-2 has been suggested to be less pathogenic than HIV-1 (31, 32). We have collected 13 HIV-2 isolates in order to compare their in vitro biological properties to those of HIV-1 isolates. Similarly to HIV-1, virus isolated from HIV-2 infected immunodeficient individuals fulfills the rapid/high criteria (33, 34). Accordingly, these viruses infect cell lines and give rise to continuous virus replication. On the other hand, HIV-2 infected asymptomatic individuals carry slow/low type of HIV-2. These viruses replicate in cell lines transiently or not at all. Still another similarity to HIV-1 is that rapid/high type of HIV-2 causes extensive syncytia formation in PBMC cultures, whereas slow/low type of HIV-2 isolates often lack this capacity. These similarities suggest that in cases where HIV-2 causes disease, albeit rarely or with longer incubation period than HIV-1, the pathogenic mechanisms operating are similar.

Infection of monocyte/macrophage cultures by HIV-1 and HIV-2.

Studies on a panel of 70 HIV-1 and 12 HIV-2 isolates have shown that all viruses can infect primary cells of the mononuclear phagocyte lineage (35). The viruses were recovered from seropositive individuals with varying severity of HIV infection, most often from the peripheral blood but also from cerebrospinal fluid, brain tissue, plasma, monocytes or CD4+ T lymphocytes. Even if the replicative capacity of the viruses varied, they all infected monocyte/macrophages, regardless of the patient's clinical stage, tissue source of isolate, repeated in vitro passage or differences in biological properties. We conclude that dual tropism for monocyte/macrophages and CD4+ T lymphocytes is a general property of HIV.

In some experiments, HIV replication in macrophages can no longer be detected one month after infection. Virus can, however, be recovered by cocultivation with PBMC (14). The virus thus recovered differs from the original isolate in replicative and cytopathic characteristics. Our results demonstrate that HIV can establish a persistent, nonproductive infection in monocyte/macrophages and, consequently, these cells may

serve as infectious resrvoir in the infected individual. Moreover, passage of HIV through macrophages may contribute to the biological diversity of virus isolates.

Biological characterization of progeny viruses obtained from transfection and cotransfection of molecular clones of a HIV-1 isolate.

In order to study the molecular determinants underlying the biological characteristics, molecular clones were obtained from a HIV-1 isolate with high replicative capacity (36). The virus isolate, 4803, has only been passaged in PBMC prior to cloning. High molecular weight DNA of 4803 infected PBMC was restricted with Xba-1 and appropriate size fragments cloned in the bacteriophage l-dash. Seven recombinant phages were identified. The clones were shown to be highly related to each other and differed only at 1 or 2 restriction sites of 26 tested. Following transfection by electroporation into various kinds of cells the phenotype of progeny viruses was shown to depend on the cell type. Viruses recovered from PBMC cultures differed from the parental isolate in that they did not form syncytia and lacked the capacity to replicate in cell lines. Since transfection of PBMC yielded progeny virus within one week, this phenotype is considered to be the true phenotype of the clones. Transfection into the T-lymphoid HUT-78 cell line and the monocytoid U937-2 cell line yielded virus after considerable delay (more than one month). Progeny viruses from HUT-78 cells were similar to the parental isolate, they formed syncytia in PBMC and replicated in all cell lines tested. Progeny viruses from U937-2 cells showed an intermediate phenotype in that they replicated in U937-2 cells but not in T-lymphoid cell lines. The results indicate that molecular clones of a rapid/high HIV-1 isolate may have restricted replicative capacity compared to the genetically heterogenous isolate. Upon infection/transfection of different cell types, diversification and selection may result in alteration of the viral phenotype in a cell type dependent manner.

To test the hypothesis that the phenotype of naturally occurring HIV-1 may be dependent on the interaction of variant viruses that are present in an isolate, cotransfection of molecular clones with slow/low replicative capacity was carried out and the progeny tested for biological properties (37). Transfection individually of each of the clones 12, 13 and 82 gave rise to slow/low viruses, whereas progeny recovered from cotransfection of the mixtures of these clones exhibited rapid/high phenotype. Further analysis of the progeny virus population by endpoint dilution revealed recombinants with high fequency. In this study, no particular biological phenotype could be linked with a certain genotype, further demonstrating the enormous complexity of linking HIV variability to genetic sequences.

Autologous neutralizing antibodies in HIV-1 and HIV-2 infection.

We have used a unique autologous system. HIV-1 has been isolated during symptomatic primary HIV-1 infection (38) and repeatedly thereafter and tested against autologous sera collected in parallel (6). Isolate-specific low-titer neutralizing antibodies developed within 2 - 4 weeks after onset of symptoms and the titers to the first isolate increased with time. In three patients we could document the emergence of virus variants with reduced sensitivity to neutralization by autol)gous, but not heterologous, sera. Recently, we and others (39-42) have shown that, in fact, in the majority of HIV-1 infected individuals (28 out of 40 tested) neutralizing antibody to simultaneously collected autologous virus cannot be detected (Table 1A). Viruses resistent to neutralization by autologous sera are not resistant to neutralization *per se*, since they can be neutralized by sera from other HIV-1 infected individuals. With time the autologous hosts mount a neutralizing antibody response to the newly emerging variants (40, 42, 44), since sera collected 10-14 months after virus isolation contain neutralizing activity in 10 out of 12 cases (Table 1B). The results suggest a mechanism by which the new virus variants evade detection by the immune system.

We have also investigated the capacity of sera from nine HIV-2 infected individuals to neutralize autologous virus isolated simultaneously with the serum sample (45). All nine HIV-2 infected individuals neutralized autologous virus with titers ranging between 20 and 320. This is in contrast to HIV-1 infection, where most individuals lack such antibodies. The difference in neutralizing antibody prevalence may explain the more rapid progression in HIV-1 than in HIV-2 infection.

Biological and antigenic variation of SIV_{sm} and HIV-2 in experimentally inoculated macaques.

It has been demonstrated previously that changes occur in the nucleotide composition of SIV_{mac} molecular clones inoculated to macaques (46), whereas inoculation of a nonpathogenic HIV-2 molecular clone (ISY) displays very limited heterogeneity (47). The nucleotide changes in SIV inoculated animals preempted changes in biological and antigenic properties of viruses in vivo. Indeed, sequential isolates obtained from SIV_{sm} infected macaques (8) differ in biological and antigenic characteristics both from the inoculum virus and from each other (43). The changes in replicative capacity and cytopathogenicity of inoculum virus and reisolates are illustrated in Table 2. In the nonpathogenic HIV-2 infection of macaques (7) no phenotypic changes occur over time (Zhang et al. unpublished). This is in line with earlier observations of Castro et al. (48) who used several HIV-2 strains for inoculation of baboons and macaques and found very

little or no host range differences between inoculum virus and reisolates in animals with asymptomatic infection.

Antigenic variation in SIVsm infection of macaques is reflected by the emergence of variant viruses resistent to neutralization by the monkeys own serum (43). The neutralizing antibody response of monkeys that succumbed with immunodeficiency within 18 months, if at all present, remained isolate-specific and decayed over time. Two long survivor monkeys developed neutralizing antibody response to a second and third reisolate, respectively, as well as to eight reisolates obtained from three other monkeys. Thus the neutralizing antibody response of the long survivor showed a relatively broad specificity one year postinfection. In contrast, in the nonpathogenic HIV-2 infection monkeys from which virus could be reisolated developed antibodies that neutralised the inoculum virus and all reisolates with equal efficiency (Zhang et al. unpublished).

Taken together, the pattern of virus neutralization in SIV infected monkeys is thus similar to HIV-1 infected humans. In both cases, variant viruses resistent to neutralization by autologous sera emerge during the entire course of infection. The ability to produce neutralizing antibodies to autologous virus appears to correlate with the degree of immunodeficiency in the host. In line with this, the genetic, biologic and antigenic variation of HIV-2 in infected macaques with no disease seems to be minimal. The animal model system thus seems to teach us that pathogenesis and variability are closely linked viral properties.

Establishment of a simple, sensitive and specific polymerase chain reaction (PCR) protocol for the detection of HIV-1 in PBMC of infected individuals.

In this test protocol (25) we amplify crude cell lysates in a two-step PCR, first with outer primers then with inner primers, nested within the first and the PCR product is visualized by agarose gel electrophoresis and ethidium bromide staining. The samples are analyzed with three (or four) sets of nested primers designed to amplify HIV-1 gag, pol and env gp41 (and env gp120) sequences, respectively. We were able to amplify HIV-1 sequences in all samples from 90 HIV-1 seropositive individuals with mostly mild symptoms. Samples from 26 healthy blood donors as well as cells infected in vitro with HIV-2 and HTLV-I were negative in PCR, thus demonstrating the specificity of the amplification. Since this technique avoids conventional DNA extraction as well as hybridization for the detection of the PCR product it may be widely used in clinical virology practice. Moreover, the use of a primer set spanning the V3 region of the envelope gp120 protein (variable region 3, also called the immunodominant loop)

allowed us to specifically study this region in neutralization resistant HIV-1 variants (described in the followings and in ref. 26).

Within the frame of studies on pediatric HIV infection we have examined the presence of HIV-1 sequences in altogether165 blood samples from 47 HIV-1 antibody positive mothers and 87 children born to HIV-1 antibody positive mothers. Early and reliable diagnosis of HIV infection in children is a particularly important issue. We were able to amplify HIV-1 sequences in samples from all mothers and all children found to be HIV-1 seropositive or showing clinical signs of immunodeficiency (49). Seronegative children or those without clinical signs of HIV-1 infection (91 samples of 76 children) were also PCR negative.

Attempts to map virus neutralizing epitopes in the HIV-1 envelope.

Synthetic peptides.

Four major sites involved in neutralization were identified by use of a panel of 80 HIV-1 antibody positive human sera (50). A correlation between high neutralization of a certain virus strain and strong reactivity with a selected homologous peptide suggested that the corresponding region might be involved in virus neutralization. In order to substantiate the role of a certain region in neutralization, peptides were used to block the neutralizing activity of selected sera. Four sites were identified, two sites on each of the envelope glycoproteins. In gp120 one was the V3 domain and the other was the carboxyl terminal end, amino acids 489-508. The latter site is conserved and was previously reported to be highly antigenic (51). Our results show that this site reacts with neutralizing antibodies. The gp41 sites encompass the previously identified, presumably intracellular, region of this molecule (amino acids 732-746) and in addition a previously unrecognized, conserved site with an external membrane position, amino acids 647-671. The results indicate that this conserved epitope of the HIV-1 envelope elicits a virus neutralizing antibody response during natural infection in humans and may therefore be considered for inclusion in a vaccine against HIV-1.

Direct DNA sequencing.

As a continuationof studies on autologous neutralization, we have analyzed the V3 region of the envelope gp120 protein (also called the immunodominant loop) of neutralization sensitive and resistant HIV-1 variants, using amplification by the polymerase chain reaction (PCR) followed by direct nucleic acid sequencing. The use of a primer set spanning the V3 region of the gp120 portion of the envelope (25) allowed us to specifically study this region (nt 7137-7572) by use of a direct solid phase DNA sequencing technique (26). The results show that in three of four individuals tested,

nucleic acid sequence of the envelope V3 region of variant viruses is identical to the V3 region of the first, neutralization sensitive isolate. In the fourth individual three point mutations were present in the V3 region 118 weeks after the onset of primary HIV-1 infection. The individual was asymptomatic at the time of the collection of this last isolate. The results indicate that resistance to neutralization by autologous antibody may arise from changes outside the V3 region. For a more detailed mapping of neutralizing epitopes within the envelope, we proceed to determine the entire nucleotide sequence of the envelope.

Recombinant human Fab fragments neutralize HIV-1.

A panel of 20 recombinant Fab fragments reacting with the surface glycoprotein of HIV-1 were examined for their ability to neutralize MN and IIIB strains of HIV-1 (52). Neutralization was determined as the ability of Fabs to inhibit infection as measured in both p24 ELISA and syncytia assays. One group of closely sequence related Fabs was found to neutralize virus in both assays at 1μg/ml. The results imply that virion aggregation or cross-linking of gp120 molecules on the virion surface are not an absolute requirement for HIV-1 neutralization. Furthermore, all of the Fabs were shown to be competitive with soluble CD4 for binding to gp120 and yet few neutralized effectively, implying that the mechanism of neutralization in this case does not involve receptor blocking. The preponderance of high affinity Fabs with poor or no neutralizing ability may have implications for vaccine strategies.

Studies on antigenic and immunogenic sites of HIV-2 glycoproteins.

Twenty five peptides representing different regions in the HIV-2$_{SBL6669}$ (clone Isy in Meyr's et al. Human retroviruses data base) envelope proteins were synthesized. The peptides were selected to represent regions which in HIV-1 had been documented to be of antigenic or immunogenic importance. Evaluation of the antigenic activity of the HIV-2 env peptides demonstrated a dominating activity in four different regions (53). These were 1) the V3 region and an adjacent (carboxy terminal) amino acid stretch located in the carboxy terminal half of the external glycoprotein, 2) the extreme carboxy terminal end of gp120, 3) a uniquely antigenic site in the amino terminal part of the transmembrane glycoprotein and 4) a preferentially HIV-2 antigenic site located further about 50 amino acids towards the carboxy terminal of the latter site.

In order to characterize the immunogenic activity of peptides, guinea pigs were immunized with KLH-coupled material. Antisera showed high ELISA titers in tests with homologous peptide antigen and reacted in about 65% of cases with intact protein as determined in whole antigen ELISA and Western blot studies. All sera were tested for

their capacity to neutralize and to mediate ADCC with the homologous virus. Seven peptides representing five different regions elicited virus neutralizing antibody activity. The highest titer was seen with sera against two overlapping peptides representing the carboxy terminal part of the V3 loop. High neutralizing activity was also found in sera against peptide Ala^{119}-Cys^{137}, Thr^{489}-Gly^{509} (with weaker activity in sera against an overlapping peptide) and His^{714}-Glu^{729}. Several anti-peptide sera showed ADCC activity but in only one case did this activity occur concomittantly with a neutralizing activity (in one of the V3-specific sera). Significant ADCC activity was also found in sera against peptides Glu^{291}-Ser^{311} and Arg^{446}-Phe^{461}.

A complete mapping of antigenic linear epitopes along the gp125 envelope protein has demonstrated that 90% of HIV-2 antibody positive human sera (31 tested) react with peptides corresponding to the V3 region of HIV-2 (54). This region is thus immunogenic in humans.

Next, we wanted to see whether the V3 region carries broadly neutralizing epitopes. We assayed the capacity of guinea pig sera raised against HIV-2 envelope peptides to mediate cross-neutralization of ten West African HIV-2 isolates (55). Antibodies to the HIV-2 V3 region cross-neutralized nine of the ten isolates tested, with titers ranging between 160-640. The neutralizing epitope(s) responsible for the high-titre broad cross-neutralizing activity was further analyzed and shown to correspond to the central and C-terminal portion of the V3 region. Using these peptides, the neutralizing activity of human HIV-2 antibody positive sera could be blocked. Two deletion sets of peptides, spanning between aa Ser^{311} and Arg^{337}, were synthesized in order to identify the role of individual HIV-2 V3 amino acids in the binding of human polyclonal and neutralizing mouse monoclonal antibodies. Two distinct antigenic sites were identified in this region, the first corresponding to the conserved motif Phe-Hys-Ser (aa 315-317), and the second in proximity of the C-terminal cysteine (Trp-Cys-Arg, aa 329-331). The results indicate that V3 is an important neutralizing domain of HIV-2 envelope. One monoclonal antibody showed dependence on both sites, signifying that they can interact to represent a local discontinuous antigenic site.

<u>Identification of four antigenic sites in the envelope proteins of SIV_{sm} recognized by macaque sera.</u>

Thirty eight overlapping peptides were synthesized and used in site-directed ELISA with sera from experimentally SIV_{sm} infected macaques (56). Four antibody-binding regions were identified, corresponding to the second variable region (V2; aa 170-196), the region homologous to V3 in HIV-1 (aa 313-346), the carboxy terminus of the major glyco-

protein (aa 514-537) and the amino terminus of the transmembrane protein (aa 608-638). The location of antigenic sites in the envelope proteins of SIV shows much resemblance to that of HIV-1 and HIV-2. Moreover, serum reactivity to the V2 region was higher in long-term survivor monkeys than in animals with a relatively early development of simian AIDS, suggesting an association between antibody reactivity to this region and disease progression.

Studies on mother-to-child transmission of HIV-1.

Neutralizing antibodies.

A pertinent question is whether neutralizing antibodies protect children born to HIV-1 infected mothers from becoming infected. Neutralizing antibodies in serum of HIV-1 infected mothers were tested against primary HIV-1 isolates obtained from the same study group (41). The results show that non-transmitting mothers more frequently have neutralizing antibodies against their own virus (called autologous virus) than transmitting mothers. In addition, all mothers with autologous neutralizing antibodies also neutralised at least two heterologous primary isolates. This suggests that broad neutralization of primary isolates (i e cross-reactive antibodies) may protect against mother-to-child transmission of HIV-1. The possibility of preventive measures by passive transfer of antibodies should be explored.

Molecular studies.

We have compared the V3 sequences from ten HIV-1 infected infants to virus sequences of the corresponding mothers (57). The infected infants, in contrast to the mothers, harboured homogenous virus populations. Comparison of sequences from the children and clones derived from DNA of the corresponding mothers showed that the transmitted virus represented either a minor or a major virus population of the mother. In contrast to an earlier study (13) we found no evidence of selection of minor virus variants during transmission. Furthermore, the transmitted virus variant did not show any characteristic molecular features.

CONCLUSIONS

The biologic properties of HIV-1 and HIV-2 are similar and correspond to the severity of the infection. In both cases, virus with slow/low replicative capacity is isolated from individuals with normal levels of CD4 cells in blood and, conversely, virus with rapid/high replicative capacity is obtained from severely immunodeficient patients. All HIV-2 isolates tested, like those of HIV-1, infect and replicate in fresh monocyte/macrophage cultures. Monocyte tropism seems to be a general property of HIV isolates. The

results indicate that the pathogenic mechanisms operating in HIV-1 and HIV-2 infection are similar. Still, the pathogenic process appears to be more prolonged in HIV-2 than in HIV-1 infected individuals. As demonstrated by our recent results, neutralizing antibodies to autologous virus are regularly present in HIV-2 infection and neutralizing antibodies show extensive cross-reactivity between isolates. Conceivably, effective virus neutralization may contribute to the delay of the pathogenic process in HIV-2 infection.

Monocyte/macrophages may have an impact on virus variability, since viruses rescued from silently infected monocyte/macrophages have different biologic properties than the infecting virus. The notion that the cell type is important in determining the biological properties of HIV has been substantiated and further extended in experiments using molecular clones of an HIV-1 isolate. The results show that transfection into different cell types alters the viral phenotype in a cell type dependent manner. Further alteration of viral phenotype may occur through recombination or complementation of the quasi-species present in one individual, as recently demonstrated by our in vitro model experiments using molecular clones.

Autologous neutralizing antibody response in HIV-1 infection is concomitant with seroconversion and shows isolate-specificity. Neutralizing antibodies seem to have a role in clearing the initial viremia since as the antibodies appear viremia disappears. However, virus isolated a few months later cannot be neutralized by the antibodies present in the patients serum indicating the emergence of neutralization resistant variants. In fact, our extended work as well as results from other groups emphasize that the lack of neutralizing antibodies to autologous virus in HIV-1 infected individuals is a general phenomenon. The results obtained in SIVsm infected macaques give further support to the notion that ability of the host to produce neutralizing antitibodies against several autologous and heterogous viral variants is associated with longer survival. In line with this, in the nonpathogenic HIV-2 infection of macaques, emergence of neutralization resistent virus variants could not be demonstrated. Sera of HIV-2 (SBL6669) infected monkeys not only neutralize autologous and heterologous reisolates, but also several other HIV-2 isolates from humans, indicating broad neutralizing antibody response.

Efforts to localize the change leading to neutralization resistance in the HIV-1 envelope have shown that the amino acid sequence of V3 region may (one of four tested) or may not change in variant viruses. Pairs of neutralization susceptible/resistent variants of SIV_{sm} do not differ in sequence of a region corresponding to V3 in HIV-1.

For the first time, immunogenic regions in the HIV-2 envelope could be mapped using synthetic peptides and guinea pig antisera. An envelope region corresponding to the V3 loop in HIV-1, gave high-titre neutralizing and ADCC-mediating antibodies. This region was also recognized by HIV-2 infected human sera. Antibodies raised in guinea pigs to peptides corresponding to the central and C-terminal part of the V3 loop were broadly cross-neutralizing many HIV-2 isolates. Our results show that the extent of cross-reactivity among HIV-2 isolates in this region of the envelope is great, probably greater than among HIV-1 isolates. Evaluation of the humoral immunity in the HIV-2 model may therefore be important for vaccine development. Successful passive immune profylaxis was already reported. Based on these results attempts will be made to boost cynomolgus macaque monkeys, which have received primary immunization with whole virus or envelope proteins, with single HIV-2 or SIV_{sm} peptides or pools of them. The identification of a unique HIV-2 V3-associated local discontinuous immunogenic site will be of considerable value in this endavour. The development of neutralizing antibodies and the susceptibility to challenge of animals with homologous virus will be determined.

Our present concepts on pathogenic mechanisms operating in HIV infection have been put forward in review articles (refs. 4 & 5). In short, the initial phase of virus replication in the infected individual may produce a wealth of variant viruses, in part by virtue of replication and in part by replication in different cell types. In the followings, low level replication of variant viruses evading the immune system may continue. Virus may escape immune detection through cell-to-cell spread, for ex. from antigen-presenting macrophages to T lymphocytes, or by emergence of antigenic escape mutants, or both. Slowly replicating viruses may therefore continue to act on cells of the immune system in a variety of ways. Whenever virus replication is slower, fewer variants will be produced. The immune system may be able to better hold in check a relatively limited number of variants, slowing down the pathogenic process. In this respect, neutralizing antibodies (and other immune defence mechanisms) would have an important role in delaying onset of clinical symptoms and mitigate the severity of infection. In addition, neutralizing antibodies may have a protective role in mother-to-child transmission of HIV-1.

Table 1. - Autologous neutralizing activity in HIV-1 infected humans and SIV_{sm} infected macaques.

A) Simultaneous samples: serum/virus isolate

	Neutralizing antibody		Reference
	absent*	present	
HIV-1 PHI/human**	0	4	Albert et al. 1990 (6)
HIV-1/human	14	2	Homsy et al. 1990 (39)
	2	2	von Gegerfelt et al. 1991 (40)
	11	7	Scarlatti et al. 1992 (41)
	1	1	Arendrup et al. 1992 (42)
SIV_{sm}/monkey	7	0	Zhang et al. manuscript (43)

B) Sequential samples: serum collected >6 months after virus isolation

	Neutralizing antibody		Reference
	none*	appearing/ increasing	
HIV-1 PHI/human**	0	4	Albert et al. 1990 (6)
	0	3	Arendrup et al. 1992 (42)
HIV-1/human	0	1	Tremblay & Wainberg 1990 (44)
	2	2	von Gegerfelt et al. 1991 (40)
	0	2	Arendrup et al. 1992 (42)
SIV_{sm}/monkey	3	4	Zhang et al. manuscript (43)

*Antibody titer <20. **PHI: primary HIV infection.

Table 2. - Biological properties of SIV_{sm} and HIV-2SBL6669 inoculum viruses and reisolates from cynomolgus macaques.

Inoculum virus			No. of animals	Reisolates >4 months PI	
	Replication in cell lines*	CPE**		Replication in cell lines*	CPE**
SIV_{sm}	yes	++	4	no	-/±
			2	no	+++
HIV-2SBL6669	yes	+	6	yes	+

*Replication in human cell lines: Jurkat, Hut-78 and U937 clone 2.

**Syncytia induction in human PBMC: +++, 50% of cells involved in syncytia formation, large syncytia in every field; ++, 10% of cells involved in syncytia formation, large syncytia rare; +, occasional syncytial cells; -, no syncytia observed.

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ANTIGEN DETECTION IS A RELIABLE METHOD FOR EVALUATING HIV/SIV NEUTRALIZATION ASSAYS

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In the August 1992 issue of AIDS Research and Human Retroviruses Burns and Desrosiers warned against using core antigen ELISA for evaluation of HIV and SIV neutralization assays (1). The authors show that some sera contain anti-gag antibodies which may interfere with the antigen detection. Such sera may give false positive neutralization results unless the anti-gag antibodies are first removed by proper washing procedures. Burns and Desrosiers indirectly imply that earlier findings published by our group may have been influenced by such interfering anti-gag antibodies. Therefore, we have critically re-examined our methods and we show here that anti-gag antibodies are unlikely to have influenced our results.

Our neutralization assays have been described in detail elsewhere. Briefly, 75µl of diluted serum (six five-fold dilutions starting with a lowest dilution of 1:10) and 75µl of diluted virus were mixed (2,3). After a 1h incubation at 37°C 100 000 blood donor peripheral blood mononuclear cells (PBMC) in 75µl of medium were added. In earlier published experiments either 180µl (2) or 100µl (3) of the culture medium was removed on each of the following three days. This resulted in final serum dilutions of at least 1:3750 and 1:180, respectively. In more recent unpublished experiments the culture medium has been removed by centrifugation on days one and three. Each centrifugation removes at least 210µl of medium which results in a final serum dilution factor of at least 1:6750. It should be noted that Burns and Desrosiers did not include any washes in their experiments.

In a first set of experiments we examined if anti-gag antibodies are capable of interfering with the in-house antigen ELISAs we use for the detection of

HIV-1 (4) and HIV-2/SIV (5). These experiments were also designed to investigate how efficient the three different washing methods are in removing such interfering antibodies. Sera with high titer anti-gag antibodies were selected and tested according to the method described by Burns and Desrosiers (figure 1). These experiments showed that anti-gag antibodies may interfere with both the HIV-1 antigen ELISA and with the HIV-2/SIV antigen ELISA. Removal of 100µl on three occasions was not sufficient to abolish the interfering activity. However, washing by centrifugation two times in the HIV-1 assay and and centrifugation once + removal of 100µl medium on two occasions (dilution fatcor 1:1720) in the HIV-2/SIV assay was sufficient to effectively remove interfering antibodies from one HIV-1 and two SIV antibody positive sera. Interfering activity could not be completely abolished from the HIV-1 serum B which contained extremely high anti-gag titer, representing less than 10% of HIV-1 positive sera (6). Tests of removal of 180 µl medium three times are under way.

Next, we investigated if the neutralizing titer of sera with and without anti-gag antibodies was influenced by the method used for washing. Table 1 and 2 show that the there was no evidence of false-positive neutralization with the least effective washing procedure (removal of 100µl x 3), not even in sera with high titer anti-gag activity. Thus, the neutralization results appeared to be unaffected although the anti-gag antibodies were not completely removed.

Last, in our previous and ongoing studies we have focused on the emergence of neutralization resistant virus variants in humans and in experimentally infected monkeys. Thus, we have repeatedly documented that sera which

neutralize early autologous isolates frequently fail to neutralize later isolates. These differences in ability of individual sera to neutralize different virus isolates can not be due to anti-gag antibodies, since such interference would not be strain-specific. It is strange that Burns and Desrosiers has illustrated the importance of anti-gag antibodies by showing that sequential sera from one monkey, which lacked anti-gag antibodies. This monkey showed different ability to neutralize autologous virus isolates. Thus, a serum obtained 102 weeks postinfection showed a neutralizing titer of 1:320 against the inoculated strain (SIVmac239), whereas the titer to a subsequent isolate was as low as 1:20. These strain-specific differences are not likely to be due to interference by anti-gag antibodies.

In summary, we have confirmed that anti-gag antibodies may interfere with the core antigen ELISAs which are commonly used to evaluate HIV and SIV neutralization assays. However, these antibodies can almost always be removed by proper washing procedures. Thus, we show that anti-gag antibodies can be removed from most sera by washing procedures that give at least an 1:6000 dilution of the serum (i.e. two cycles of centrifugation of microtiter plates). Furthermore, even if interfering antibodies may be detected in specially designed interference assays this does not mean that anti-gag antibodies will affect the neutralization assay. In conclusion, the use of antigen detection is a safe, efficient and rapid method for determining neutralizing antibody titers in sera of HIV infected individuals and SIV infected monkeys.

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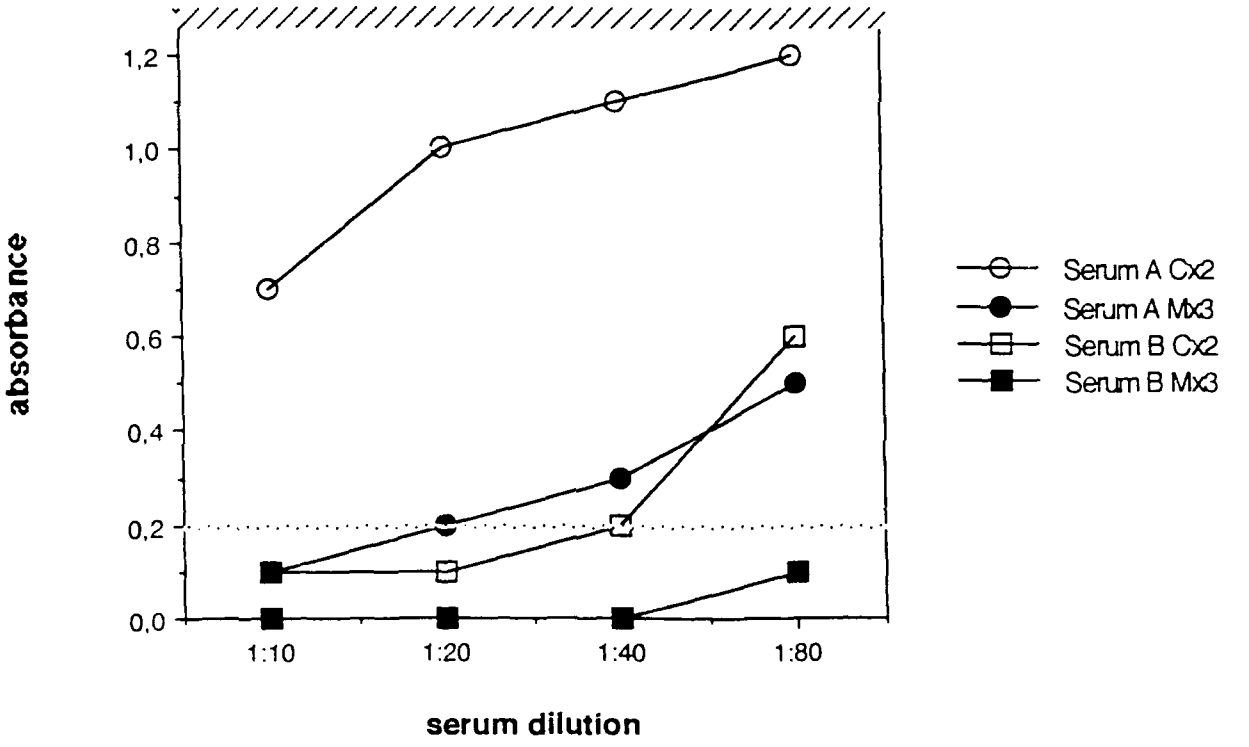
FIGURE LEGENDS

Figure 1. Capacity of selected sera, with high titer anti-gag antibodies, to interfere with core antigen detection by ELISA. The assays were performed according to Burns and Desrosiers (1), with minor modifications. 75 µl of each serum dilution was mixed with 100 000 PBMC in 150 µl volume in 96-well plates. Starting on day one the plates were washed according to the following protocol. HIV-1: Cx2, wash by centrifugation on day 1 and 3; Mx3, change of 100µl medium on day 1,2, and 3. SIV: wash by centrifugation on day 1, followed by change of 100µl medium on day 2 and 3. Supernatants were harvested on day 1 before the first medium change and on day 3 after the last medium change. The ability of the supernatants to interfere with the detection was then investigated. Known amounts of core antigen (1 ng/ml) was added to the supernatants, which were then tested by HIV-1 (4) or HIV-2/SIV (5) antigen ELISAs. Serum A and B were obtained from HIV-1 seropositive asymptomatic individuals who tested strongly positive for anti-gag antibodies (Abbott core EIA) (table 1). Serum M10:8 and H28:8 were obtained from two cynomolgus monkeys 225 and 125 days, respectively, after experimental infection with SIVsm (7). Both monkey sera had an anti-gag titer of 1:1000 when tested in ELISA utilizing recombinant SIV gag protein as antigen.

HIV-1


absorbance
1,2
1,0
0,8
0,6
0,4
0,2
0,0
1:10
1:20
1:40
1:80
serum dilution
Serum A Cx2
Serum A Mx3
Serum B Cx2
Serum B Mx3

SIV

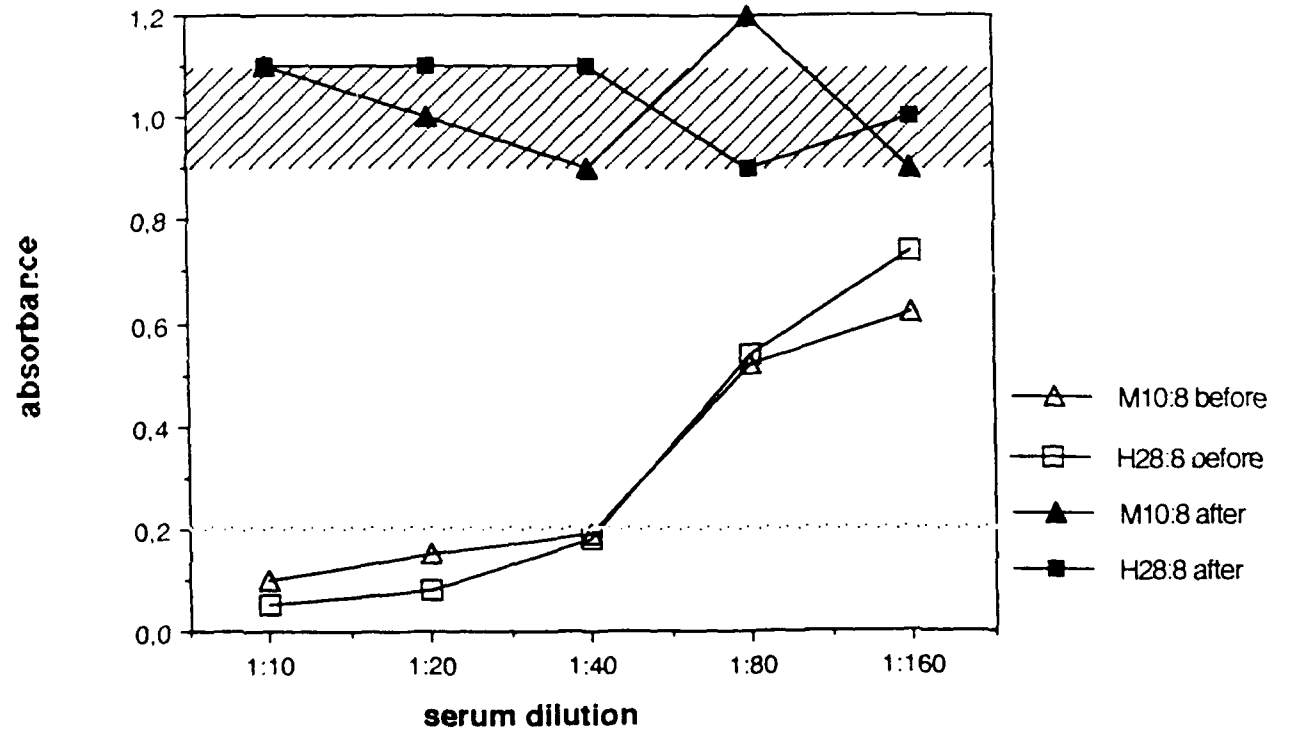

absorbance
1,2
1,0
0,8
0,6
0,4
0,2
0,0
1:10
1:20
1:40
1:80
1:160
serum dilution
M10:8 before
H28:8 before
M10:8 after
H28:8 after

Table 1. - The washing procedure has no effect on virus neutralization titer of HIV-1 sera with varying anti-gag antibody content.

Serum	anti-gag activity ratio*	ID-50 used	Number of tests	Neutralizing titer	
				washing by 2 cycles of centrifugation **	medium change 100µl x3 ***
A	32	75	2	<20	20
B	74	50	2	<20	<20
C	37	50	2	20	<20
D	<1	10	1	160	40
E	<1	50	1	<20	<20
		10	1	<20	<20

*Abbott core EIA; ratio = $\frac{\text{absorbance value of cut off}}{\text{absorbance value of sample}}$

**Used by Scarlatti et al. unpublished. ***Used by von Gegerfelt et al. 1991.

Table 2. - The washing procedure has no effect on virus neutralization titer of HIV-2 sera with varying anti-gag antibody content.

serum	anti-gag antibody in serum*	anti-gag antibody in supernatant		virus neutralizing titer of serum	
		washing by 3 cycles of centrifugation **	medium change 150 µlx3	washing by 3 cycles of centrifugation	medium change 150 µl x3
A2	0.853	0.003	0.032	40	40
B2	3.000	0.077	0.667	80	80

*Absorbance value of a 1:100 dilution of serum tested against recombinant HIV-2 gag protein in ELISA.

**Used by Björling et al. unpublished.